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DNA Methylation in Newborns and Maternal Smoking in Pregnancy: Genome-wide Consortium Meta-analysis

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Epigenetic modifications, including DNA methylation, represent a potential mechanism for environmental impacts on human disease. Maternal smoking in pregnancy remains an important public health problem that impacts child health in a myriad of ways and has potential lifelong consequences. The mechanisms are largely unknown, but epigenetics most likely plays a role. We formed the Pregnancy And Childhood Epigenetics (PACE) consortium and meta-analyzed, across 13 cohorts ($n = 6,685$), the association between maternal smoking in pregnancy and newborn blood DNA methylation at over 450,000 CpG sites (CpGs) by using the Illumina 450K BeadChip. Over 6,000 CpGs were differentially methylated in relation to maternal smoking at genome-wide statistical significance (false discovery rate, 5%), including 2,965 CpGs corresponding to 2,017 genes not previously related to smoking and methylation in either newborns or adults. Several genes are relevant to diseases that can be caused by maternal smoking (e.g., orofacial clefts and asthma) or adult smoking (e.g., certain cancers). A number of differentially methylated CpGs were associated with gene expression. We observed enrichment in pathways and processes critical to development. In older children (5 cohorts, $n = 3,187$), 100% of CpGs gave at least nominal levels of significance, far more than expected by chance (p value $< 2.2 \times 10^{-16}$). Results were robust to different normalization methods used across studies and cell type adjustment. In this large scale meta-analysis of methylation data, we identified numerous loci involved in response to maternal smoking in pregnancy with persistence into later childhood and provide insights into mechanisms underlying effects of this important exposure.

Introduction

Despite years of advisories regarding health risks to the developing fetus from maternal smoking, many pregnant women still smoke, including 12.3% in the US.¹ Maternal

smoking during pregnancy is regarded as a cause of low birth weight, reduced pulmonary function (PLF [MIM: 608852]), orofacial clefts (OFC1 [MIM: 119530]), and sudden infant death syndrome (SIDS [MIM: 272120]) in exposed newborns.² Other adverse birth outcomes³ have

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been associated with maternal smoking in pregnancy, along with common health problems in children, including asthma (ASRT [MIM: 600807]), otitis media (OMS [MIM: 166760]), and neurobehavioral disorders.²

The mechanisms for the adverse health effects of maternal smoking during pregnancy on offspring remain poorly understood.² Recently, studies have examined the potential role of epigenetic modifications such as DNA methylation at specific CpG sites (CpGs). These include studies examining genome-wide DNA methylation in newborns in relation to maternal smoking in pregnancy with the Illumina Infinium HumanMethylation27 (27K) BeadChip^{4–6} or the newer platform with wider coverage, the HumanMethylation450 (450K) BeadChip.^{7–10} A number of differentially methylated loci have been identified in offspring in relation to maternal smoking in pregnancy in individual studies (references in the [Supplemental](#)

[Note](#)). One study examined the top CpGs with respect to timing of exposure and found that the signals reflect sustained, rather than short-term, exposure to maternal smoking during pregnancy,¹¹ but this has not been evaluated genome-wide. A few studies suggest that some of these methylation signals persist into later childhood and adolescence, but data are limited.^{9,12} The combination of genome-wide data across studies via meta-analysis to generate large sample sizes for the discovery of loci that would not have been identified from individual studies has been very successful in genetics, but this approach has rarely been used with methylation data.

To address the impact of maternal smoking during pregnancy on newborns with much greater power, we recruited 13 birth cohort studies with data on maternal smoking during pregnancy and DNA methylation in offspring from the 450K BeadChip into the Pregnancy and

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Childhood Epigenetics consortium (PACE). We meta-analyzed harmonized cohort-specific associations between maternal smoking during pregnancy and DNA methylation in the offspring. We examined both sustained maternal smoking and any smoking during pregnancy. We also examined persistence of DNA methylation patterns related to maternal smoking in newborns among older children, including adjustment for postnatal secondhand tobacco smoke exposure. For functional follow-up of findings, we evaluated the associations between methylation status in the newly identified CpGs and expression levels of nearby genes and performed pathway and functional network analyses. This study represents a large and comprehensive evaluation of the impact of maternal smoking during pregnancy on DNA methylation in offspring.

Material and Methods

Participating Cohorts

A total of 13 PACE cohorts participated in the meta-analysis of maternal smoking during pregnancy and 450K DNA methylation in newborns. These studies, listed in alphabetical order, are the Avon Longitudinal Study of Parents and Children (ALSPAC), the Center for Health Assessment of Mothers and Children of Salinas (CHAMACOS), the Children's Health Study (CHS), the GECKO Drenthe cohort, the Generation R Study, Isle of Wight (IOW), Mechanisms of the Development of Allergy (MeDALL), three independent datasets from the Norwegian Mother and Child Cohort Study (MoBa1, MoBa2, and MoBa3), the Norway Facial Clefts Study (NFCS), the Newborn Epigenetics Study (NEST), and Project Viva. MeDALL represents a pooled analysis of four cohorts with coordinated methylation measurements: Infancia y Medio Ambiente (INMA), Etudes des Déterminants pré et postnatals précoces du développement et de la santé de l'Enfant (EDEN), Children's Allergy Environment Stockholm Epidemiology study (BAMSE), and Prevention and Incidence of Asthma and Mite Allergy (PIAMA). Two of the MeDALL cohorts contributed to the newborn meta-analysis (INMA and EDEN). There were five studies with data on older children: ALSPAC, Genes-environments and Admixture in Latino Americans (GALA II), the Study to Explore Early Development (SEED), MeDALL (INMA, EDEN, BAMSE, and PIAMA), and an independent methylation dataset from BAMSE subjects. Ethical approval for study protocols was obtained for all participating cohorts. Further information on this as well as the study methods for each cohort are described in detail in the [Supplemental Note](#).

For this paper, participating cohorts shared only results files from in-house analyses. No individual data were shared for this paper. Therefore, access to the individual cohort-level data for the purpose of reproducing results would require individual data transfer agreements to be negotiated with and approved by each of the contributing cohorts.

Harmonization of Maternal Smoking Variables

Cohorts assessed maternal smoking during pregnancy via questionnaires completed by the mothers. The MoBa study (MoBa1 and MoBa2) also used cotinine measurements from maternal blood samples taken during pregnancy as part of the definition

of maternal smoking during pregnancy. More details on the cohort-specific smoking variables are in the [Supplemental Note](#). In a previous publication from the MoBa1 study, significant associations between maternal smoking during pregnancy and DNA methylation in newborns were driven not by transient smoking that ended early in pregnancy but rather by sustained smoking during pregnancy.¹¹ Thus, each cohort ran separate models to evaluate both sustained smoking and any smoking during pregnancy. The variable (yes/no) for sustained smoking during pregnancy was designed to capture women who smoked at least one cigarette per day through most of pregnancy. To cleanly contrast the effect of sustained smoking through pregnancy with that of never smoking during pregnancy, we excluded women who reported quitting smoking during pregnancy from the sustained smoking models. The variable (yes/no) for any maternal smoking during pregnancy was designed to capture any amount of smoking during pregnancy, at any time, even if a woman reported quitting. Because we did not exclude women who quit smoking during pregnancy from the models representing any smoking during pregnancy, the total sample sizes are slightly larger than those of the models representing sustained smoking during pregnancy. Genome-wide analyses use large sample statistics. We limited meta-analyses to cohorts with at least 15 subjects in both the exposed and unexposed groups. This excluded four cohorts (CHAMACOS, CHS, IOW, and Project Viva) from the sustained smoking models. However these cohorts did participate in the meta-analysis of any smoking during pregnancy.

Methylation Measurements and Quality Control

Each cohort independently conducted laboratory measurements and quality control. The samples for each cohort underwent bisulfite conversion via the EZ-96 DNA Methylation kit (Zymo Research). Samples were processed with the Illumina Infinium HumanMethylation450 (450K) BeadChip (Illumina) at Illumina or in cohort-specific laboratories.

Quality control of samples was performed by each cohort and failed samples were excluded on the basis of Illumina's detection p value, low sample DNA concentration, sample call rate, CpG-specific percentage of missing values, bisulfite conversion efficiency, gender verification with multidimensional scaling plots, and other quality control metrics specific to cohorts. Cohorts could also use validated, published statistical methods for normalizing their methylation data on the untransformed methylation beta values (ranging from 0 to 1). Some cohorts also made independent probe exclusions. More details are provided in the [Supplemental Note](#). For the meta-analysis, additional probe exclusions were made across all cohorts. Specifically, we excluded control probes ($n = 65$), probes that mapped to the X ($n = 11,232$) or Y ($n = 416$) chromosomes, probes with an underlying SNP mapping to the last five nucleotides of the probe sequence ($N = 9,168$) as previously described,⁷ and CpGs with an implausible (zero) value for the SE ($n = 67$). This left a total of 464,628 CpGs included in the meta-analysis.

Cohort-Specific Statistical Analyses

Each cohort ran independent statistical analyses according to a common pre-specified analysis plan. Robust linear regression was used in R¹³ to evaluate the association between maternal smoking during pregnancy and cord blood DNA methylation for each probe while accounting for potential heteroskedasticity and/or influential outliers. Each cohort ran the following

covariate-adjusted statistical models: (1) the primary model, which used sustained maternal smoking during pregnancy as the exposure and the normalized betas as the outcome, (2) sustained maternal smoking during pregnancy as the exposure and raw betas (not normalized) as the outcome, (3) any maternal smoking during pregnancy as the exposure and normalized betas as the outcome, (4) any maternal smoking during pregnancy as the exposure and raw betas as the outcome, and (5) sustained maternal smoking during pregnancy as the exposure and normalized betas as the outcome, with additional adjustment for cell type proportion. All models were adjusted for maternal age, maternal education (or a surrogate socioeconomic metric), parity, and technical covariates such as batch or plate. Some cohorts used ComBat¹⁴ to account for batch effects and therefore did not include batch or plate as covariates in the models with normalized betas (see [Supplemental Note](#)). Additional correction for study design or sampling factors was done as needed in some cohorts. Because maternal smoking during pregnancy is not related to the child's sex, it cannot be a confounder and thus was not included in models. We did not adjust for principal components (PCs) because not all cohorts had genome-wide genotype data and cohorts with genotype data had it only for a subset of subjects with methylation data. Furthermore, in one large cohort with PC data, models adjusted for PCs showed little variation in the results (correlation of betas = 0.991; correlation of log(p values) = 0.996) when compared to models without this adjustment, despite a reduction in sample size. The statistical models for cohorts with DNA methylation measured in older children were the same, with the additional adjustment for second-hand tobacco smoke exposure.

All cohorts independently estimated cell type proportion by using the reference-based Houseman method¹⁵ in the minfi package¹⁶ with the Reinius et al. dataset for reference.¹⁷ Cell type correction was applied by inclusion of the six estimated cell type proportions (CD8T, CD4T, NK cells, B cells, monocytes, granulocytes) as covariates in cohort-specific statistical models.

Meta-analysis

We performed inverse variance-weighted fixed-effects meta-analysis with METAL.¹⁸ We accounted for multiple testing by controlling the false discovery rate (FDR) at 5%, implementing the method by Benjamini and Hochberg.¹⁹ This method was applied to all instances of FDR correction described in this paper unless otherwise specified. CpGs with an FDR-corrected p value less than 0.05 were considered statistically significant. CpGs that were statistically significant based on the more stringent Bonferroni correction (uncorrected p value < 1.08×10^{-7} to account for 464,628 tests) were also noted.

To determine the robustness of our models and findings, we performed an additional analysis in which we removed the cohorts of non-European ancestry ([Table S1](#)). We compared the effect estimates, SEs, and the distribution of the p values for the model to the estimates for our primary model to evaluate the consistency of our findings.

Examination in Older Children of CpGs Associated with Smoking in Cord Blood

The FDR-significant CpGs identified in the primary model from the newborn meta-analyses were followed up with a lookup replication approach in the results from five older children cohorts, and FDR correction was applied to account for the number of CpGs tested.

Literature Review to Identify Genes Previously Associated with Smoking and Methylation

We performed a systematic literature review to determine which CpGs represented findings not previously related to smoking exposure and methylation in the literature. A query of NCBI's PubMed database was performed with the search terms ("DNA Methylation"[Mesh] OR methylation) AND ("Smoking"[Mesh] OR smoking)) in order to be broad enough to capture all past studies reporting such results. CpGs with previously reported associations with smoking, both from prenatal exposure or in adults, were considered. This search yielded 789 results when performed on March 1, 2015. All results were then reviewed by title and abstract to determine whether they met inclusion criteria. First, results were limited to those performed in healthy human populations. That is, participants could not exclusively have been drawn from disease cases and studies could not have been performed only in cell lines or animals. Case-control analyses that included healthy controls were accepted as meeting this criterion, and no limitation was applied concerning the tissue used for DNA extraction. Second, studies were required to have performed DNA methylation analysis agnostically on a large scale as opposed to targeted interrogation of candidate CpGs. This was operationalized by including only analyses that examined >1,000 sites simultaneously. The Illumina 450K, 27K, and GoldenGate arrays all met this criterion. Third, the exposure was restricted to tobacco cigarette smoking. Related exposures, such as to other forms of tobacco use or smoke exposure, were not included. Lastly, studies had to have reported their significant results publicly. Studies that failed to report p values or gene annotations were excluded.

Review of the existing literature on the effect of smoking on DNA methylation identified 25 publications meeting inclusion criteria. Of these, 16 studies reported results for adult smoking exposure,^{20–35} and nine provided results of association between maternal smoking during pregnancy on child DNA methylation.^{4,7–9,12,36–39} CpG level results (p values and gene annotations) for sites showing significant association between smoking exposure and DNA methylation were extracted and compiled for comparison with the results from the meta-analysis. Results were considered significant if they met the multiple testing criteria implemented within the publication. For studies failing to implement any multiple testing correction, a naive Bonferroni threshold for the number of tests performed in the individual study was used. Genes previously associated with either adult smoking or maternal smoking in pregnancy ([Table S6](#)) were excluded from our list of meta-analysis results.

CpG Annotation

The official gene name was noted for each CpG via Illumina's genome coordinate.⁴⁰ We enhanced the annotation provided by Illumina by using the UCSC Genome Browser (including data the RefSeq and Ensembl databases), as well as annotation data in Bioconductor. All of the annotations use the human February 2009 (GRCh37/hg19) assembly. We also used the program Snipper to annotate the nearest genes within 10 Mb of each CpG. We include this expanded Snipper gene annotation in our tables and the [Discussion](#).

For selected genes, we used coMet⁴¹ to graphically display additional information about CpGs, including physical location, correlation, statistical significance, and functional annotation.

Enrichment Analysis

We evaluated whether the CpGs significantly associated with smoking (based on the FDR p value < 0.05) were enriched, relative to all CpGs analyzed, for several biologic annotations provided in the Illumina annotation file. We assessed enrichment by using the two-sided doubling mid p value of the hypergeometric test.²⁴ We also evaluated enrichment in a subset of CpGs mapping to imprinted differentially methylated regions (DMRs) described by Court et al.⁴²

Pathway Analyses

We linked the CpGs significantly associated with smoking (based on an FDR p value < 0.05) to genes on the basis of only the 450K BeadChip annotation file.⁴³ Probes lacking an annotated Entrez Gene ID were filtered ($n = 1,971$), as were duplicate gene entries ($n = 1,473$). A total of 2,629 unique gene identifiers were used in gene ontology enrichment analysis with three different procedures as described below.

This resulted in 2,235 genes that mapped to gene ontologies of biological processes, and using topGO in R,⁴⁴ we tested for gene enrichment over the background array (16,119 unique annotated Entrez Gene IDs) by using Fisher's exact tests with a minimum of five genes per node. In addition, we used the DAVID bioinformatics resource⁴⁵ to test for enrichment in gene ontology biological processes with a threshold of five, and we used the Benjamini-Hochberg procedure to control for false discoveries. Finally, we used QIAGEN's Ingenuity Pathway Analysis (IPA) to identify relevant signaling and functional pathways.

Functional Network Analysis

To construct a functional association network, it was desirable to reduce the list of tested CpGs, so we prioritized the FDR-significant CpGs from the primary model in a stepwise manner. First, we only included those CpGs that were FDR significant in both the primary and cell-type-adjusted models (FDR p values < 0.05). Next, we sorted these CpGs according to their effect size (beta coefficient), and selected the top quartile ($n = 980$). The genes mapping to these prioritized CpGs were then used as input for the construction of a functional interaction network. We used the GeneMANIA algorithm, as well as its functional association data, including genetic interaction, physical interactions, co-expression, shared protein domains, and co-localization networks.⁴⁶ We selected the "all available networks" option with a 500-gene output (accessed March 11, 2015). Functional enrichment analysis was then performed on all genes from the constructed interaction network against Gene Ontology (GO) terms to detect significantly enriched GO terms.⁴⁷ FDR correction was applied to this analysis based on the q value; a threshold of $q < 0.01$ was used.

Methylation Transcription Analysis

To further explore the associations between methylation and gene expression, we performed methylation-expression analyses, evaluating the association between the methylation status of CpGs and differences in quantitative levels of gene expression. All identified CpGs that reached FDR-corrected significance and that we identified as not previously reported in the literature were tested for association with expression levels of genes within a region of 250 kb upstream or downstream of the CpG⁴⁸ (total region 500 kb) to evaluate whether the CpG-methylation status influenced transcript levels of genes. We had two datasets available for this analysis. One dataset included mRNA gene expression (Illumina

HumanHT-12 v.4) and 450K methylation data, both from whole-blood samples from 730 adults over 45 years of age in the Rotterdam Study, a population-based prospective cohort study in Rotterdam, the Netherlands.⁴⁹ This gene expression dataset is available at the GEO public repository under the accession number GEO: GSE33828. The second dataset included mRNA gene expression (Affymetrix Human Transcriptome Array 2.0) and 450K methylation data on whole-blood samples from 107 children at 4 years of age from the INMA study in Spain. Study population details for the Rotterdam Study and INMA are in the [Supplemental Note](#). In the Rotterdam dataset, 2,636 of the 2,965 CpGs examined mapped to a transcript within the 500 kb window. We created residuals for mRNA expression after regressing out the Houseman-estimated white-blood-cell proportions, the erythrocyte and platelet cell counts, fasting state, RNA quality score, plate number, age, and sex on the mRNA expression levels by using a linear mixed model. We then created residuals for DNA methylation, regressing out the Houseman-estimated white-blood-cell proportions, age, sex, batch effects on the dasen-normalized⁵⁰ beta-values of the CpGs by using a linear mixed model. We used a linear regression model to evaluate the association between the residuals of the mRNA expression levels and the residuals of the dasen-normalized beta-values of the CpGs.

The INMA gene expression data were normalized with Expression Console Software from Affymetrix, and probes were clustered to the transcript level. Only transcripts within the 500 kb window of selected CpGs were considered in the analysis ($n = 45,076$ transcripts). To control for technical variation in the DNA methylation dataset, a PC analysis of 600 negative control probes was performed with 10,000 permutations, and the residuals of a linear regression model including the first five PCs were estimated. The effect of sex and Houseman cell-type-proportion estimates were adjusted for in a second-stage linear regression model. Two models were applied to control for technical and unwanted biological variation when estimating gene expression residuals. In the first one, sex and Houseman estimates were regressed out. In the second one, 14 surrogate variables estimated with the sva R package¹⁴ were adjusted for in a second model including sex and Houseman estimates. A linear regression model of residuals of gene expression versus residuals of methylation was performed. Multiple testing for both Rotterdam and INMA gene expression analyses was controlled with Benjamini-Hochberg FDR correction.

Examination of Polymorphic and Cross-Reactive Probes

The list of FDR-significant CpGs was matched to the list of polymorphic and cross-reactive CpGs provided by Chen et al.⁵¹ to identify potential problematic probes. We additionally performed the dip test⁵² for unimodality for each CpG to test for non-unimodal distributions in the MoBa1 cohort ($n = 1,068$). Also using the MoBa1 cohort, we visually inspected density plots for each of the probes that matched to the list of polymorphic probes from Chen et al. to assess departures from unimodality, including from small numbers of outlier values.

Results

Study Characteristics

A total of 13 cohorts participated in the meta-analysis of maternal smoking during pregnancy and 450K DNA

Table 1. Smoking Variable Frequencies for the Cohorts Participating in Meta-analyses: Newborns and Older Children

Study ^a	Study Population	Participants ^b	No. of Participants Exposed to Sustained Maternal Smoking during Pregnancy (%)	No. of Participants Exposed to Any Maternal Smoking during Pregnancy (%)
ALSPAC	newborns	860	87 (10.1)	120 (14.0)
CHAMACOS	newborns	378	7 (1.9) ^c	24 (6.3)
CHS	newborns	85	NA ^c	22 (25.9)
GECKO	newborns	255	70 (27.5)	129 (50.6)
Generation R	newborns	883	129 (14.6)	220 (24.9)
IOW	newborns	90	9 (10.0) ^c	23 (25.6)
MeDALL	newborns	362	43 (11.9)	63 (17.5)
MoBa1	newborns	1,063	156 (14.7)	312 (29.4)
MoBa2	newborns	671	70 (10.4)	173 (25.8)
MoBa3	newborns	252	28 (11.1)	73 (29.0)
NEST	newborns	413	69 (16.7)	136 (32.9)
NFCS	newborns	889	245 (27.6)	325 (36.6)
Project Viva	newborns	485	14 (2.9) ^c	26 (5.4)
ALSPAC	older children	840	89 (10.6)	115 (13.7)
BAMSE	older children	347	26 (7.5)	43 (12.4)
GALA II	older children	569	40 (7.0)	76 (13.4)
MeDALL	older children	851	86 (10.2)	121 (14.3)
SEED	older children	584	25 (4.3)	49 (8.4)

NA, not available.

^aStudy names and additional information: The Avon Longitudinal Study of Parents and Children (ALSPAC), the Center for Health Assessment of Mothers and Children of Salinas (CHAMACOS), the Children's Health Study (CHS), the GECKO Drenthe cohort, the Generation R Study, Isle of Wight (IOW), Mechanisms of the Development of Allergy (MeDALL), three independent datasets from the Norwegian Mother and Child Cohort Study (MoBa1, MoBa2, and MoBa3), the Norway Facial Clefts Study (NFCS), the Newborn Epigenetics Study (NEST), and Project Viva. MeDALL represents a pooled analysis of four cohorts with coordinated methylation measurements: Infancia y Medio Ambiente (INMA), Etudes des Déterminants pré et postnatals précoces du développement et de la santé de l'Enfant (EDEN), Children's Allergy Environment Stockholm Epidemiology study (BAMSE), and Prevention and Incidence of Asthma and Mite Allergy (PIAMA). Two of the MeDALL cohorts contributed to the newborn meta-analysis (INMA and EDEN). Studies with data on older children: ALSPAC, Genes-environments and Admixture in Latino Americans (GALA II), the Study to Explore Early Development (SEED), MeDALL (INMA, EDEN, BAMSE, and PIAMA), and an independent methylation dataset from BAMSE subjects.

^bNumber of participants with smoking data, 450K methylation, and covariates. Participants who quit smoking during pregnancy were not included in the sustained smoking models.

^cCohorts in which the sustained smoking category had $n < 15$ or insufficient information to create the requested category, resulting in exclusion from the sustained smoking analysis models. All cohorts were included in the models evaluating the exposure of any smoking during pregnancy.

methylation in newborns. Among these 6,685 newborns, 897 (13%) were exposed to sustained maternal smoking during pregnancy and 1,646 (25%) were exposed to any maternal smoking during pregnancy. We also included five cohorts of older children ($n = 3,187$, average age = 6.8 years); 266 children (8%) were exposed to sustained smoking during pregnancy and 404 (13%) were exposed to any maternal smoking during pregnancy. The cohort-specific summary statistics for maternal smoking are presented in Table 1 and covariates in Table S1. The majority of participants were of European ancestry (Table S1).

Meta-analysis

Our primary model evaluated the association between sustained maternal smoking during pregnancy and differential DNA methylation in newborns by using normalized methylation betas as the outcome, adjusting for covariates

(Figure 1). The cohort-specific lambdas and number of CpGs included in each model are listed in Table S2. Among the 6,073 CpGs with FDR significance (Table S3), 568 also met the strict Bonferroni threshold for statistical significance (p value $< 1.08 \times 10^{-7}$, correcting for 464,628 independent tests). Results were quite robust to cell type adjustment (Table S3): all 568 Bonferroni-significant CpGs from the primary model remained FDR significant in the cell-type-adjusted model, and 78% were Bonferroni significant in both models. The $\log_{10}(p$ values) for the primary model and cell-type-adjusted models were highly correlated (correlation coefficient = 0.92 across all CpGs, 0.98 for the FDR-significant CpGs in the primary model, Figure S1). Given the general similarity of the results before and after cell type adjustment and the fact that the available reference panel is from a small number ($n = 6$) of adult men,¹⁷ we regard the covariate-adjusted model as the

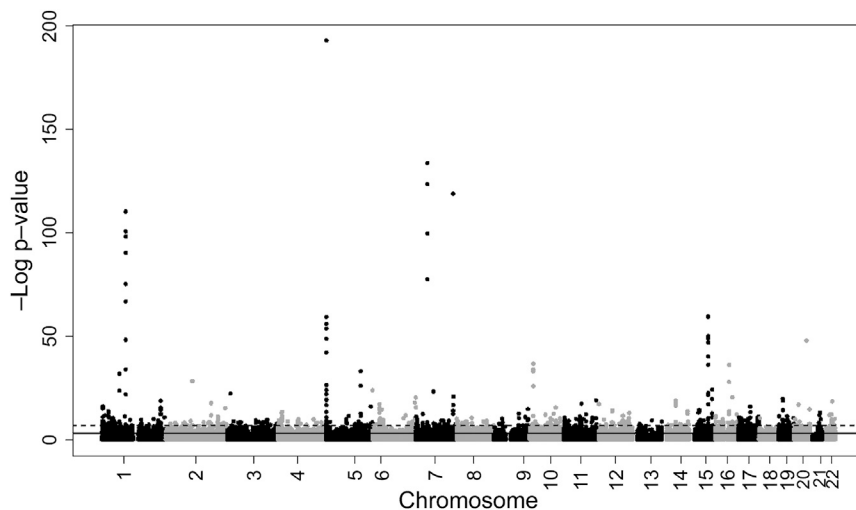


Figure 1. Meta-analysis of the Association between Sustained Maternal Smoking during Pregnancy and DNA Methylation in Newborn Cord Blood

A total of 6,073 CpGs were considered statistically significant when using FDR correction (solid horizontal line); 568 were Bonferroni significant (dashed horizontal line).

Examination of Potentially Polymorphic and Cross-Reactive Probes

A total of 742 of the 6,073 FDR-significant CpGs overlapped with the list of 70,889 potentially polymorphic probes provided by a table of Chen et al.⁵¹ Only 137 of the 6,073 FDR-significant CpGs overlapped with the list of 29,233 cross-reactive probes annotated by Chen et al. Many of the probes flagged by Chen et al. are associated with very low-frequency SNPs and thus are likely to have minimal impact on results in most datasets. In visual inspection of the density plots of all 742 such probes, we flagged 19 CpGs as having a possible deviation from unimodality (listed in Table S7). However, results from the dip test⁵² applied to all 6,073 FDR significant CpGs identified only four CpGs as statistically significantly deviated from unimodality (FDR adjusted $p < 0.05$; cg11459648, cg17847044, cg15028160, and cg25849281).

primary model. The results for other models (the cell-type-adjusted model, the model representing any smoking during pregnancy, and the methylation model representing sustained smoking during pregnancy associated with older children) and the mean methylation values in newborns and older children are shown for all 6,073 CpGs in Table S3.

Among the 6,073 FDR-significant CpGs, smoking during pregnancy was associated approximately equally with increased methylation (52%) and decreased methylation (48%) (Figure 2). Out of the 3,932 CpGs that were also FDR-significant after cell type adjustment, there were 967 CpGs in or within 10 Mb of the 1,185 genes we identified in our systematic literature review (see Supplemental Note and Table S4) as previously reported to be differentially methylated in relation to smoking. This left 2,965 CpGs (corresponding to 2,017 annotated mapped or nearest genes) that had not previously been reported (Table S5; genes highlighted in discussion shown in Table 2). For comprehensive comparison with the previous literature, we also present our results for all CpGs that were either not FDR significant after cell type adjustment and/or that annotated to genes already described in the literature as related to smoking and methylation ($n = 3,108$ CpGs, Table S6). Our top finding among the 6,073 FDR-significant CpGs was for *AHRR* (MIM: 606517) cg05575921 (p value = 1.64×10^{-193}), which is the top most statistically significant CpG in many other studies evaluating either personal smoking or maternal smoking during pregnancy.

We found our results to be robust to different analytic approaches. We present results from models using normalized betas as the outcome. When using raw betas as the outcome, we observed little difference in the results (Spearman's correlation coefficient = 0.96 for regression coefficients; 0.98 for \log_{10} [p values] for our significant findings). Furthermore, exclusion of the one cohort with newborns of non-European ancestry (NEST) from the model representing sustained maternal smoking provided similar results (Spearman's correlation coefficient = 0.99 for regression coefficients; 0.89 for \log_{10} [p values]).

nificant CpGs overlapped with the list of 29,233 cross-reactive probes annotated by Chen et al. Many of the probes flagged by Chen et al. are associated with very low-frequency SNPs and thus are likely to have minimal impact on results in most datasets. In visual inspection of the density plots of all 742 such probes, we flagged 19 CpGs as having a possible deviation from unimodality (listed in Table S7). However, results from the dip test⁵² applied to all 6,073 FDR significant CpGs identified only four CpGs as statistically significantly deviated from unimodality (FDR adjusted $p < 0.05$; cg11459648, cg17847044, cg15028160, and cg25849281).

Persistence in Older Children of DNA Methylation Related to Maternal Smoking during Pregnancy

Because of the smaller sample size and smaller proportion of children exposed to maternal smoking in the older children models, we had less statistical power than we did for the newborn models. When we compared the coefficients for newborns and older children for all 6,073 CpGs that were significantly associated with maternal smoking in newborns, 4,403 (73%) had a consistent direction of effect and all 6,073 (100%) gave nominal p values < 0.05 for the older children models, which is higher than the 5% expected by chance alone (Kolmogorov p value $< 2.2 \times 10^{-16}$). Among these, 3,722 CpGs (61%) had a weaker effect size (attenuation) in the older children than in the newborns, but the attenuation overall was very small in magnitude and not significant (mean attenuation = -0.00039 , $SD = 0.0059$). Compared to CpGs in newborns, of the 148 CpGs that met FDR significance at lookup replication level in the older children (Table S8), 100% were consistent in the direction of effect, and there was attenuation for 32%, again small in magnitude and not significant (mean attenuation = -0.00008 , $SD = 0.018$).

Enrichment Analysis

For our 6,073 FDR-significant CpGs, we observed enrichment for localization to CpG island shores (35% versus

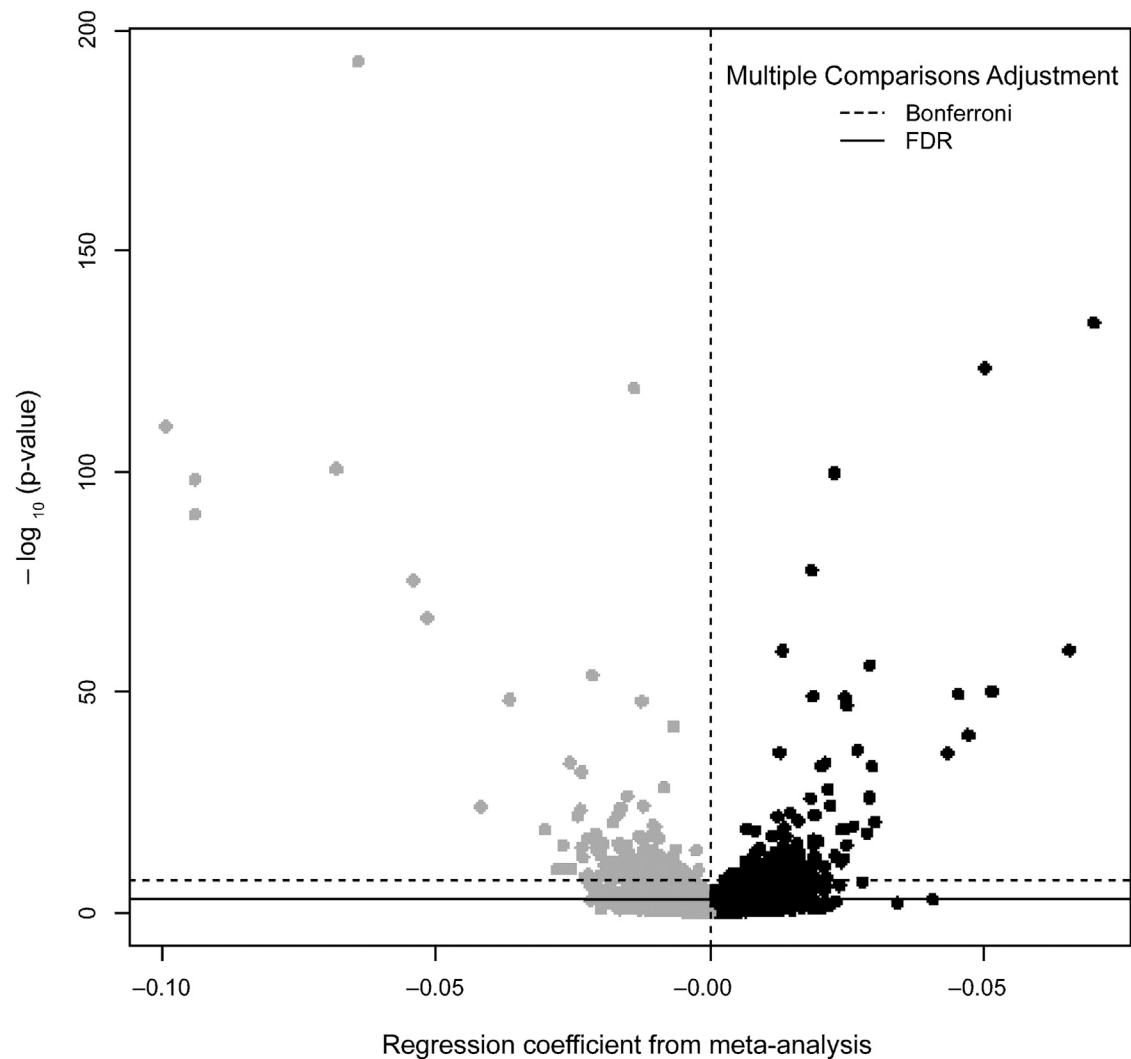


Figure 2. Volcano Plot Indicating the Direction of Effects for the Meta-analysis of the Association between Sustained Maternal Smoking during Pregnancy and DNA Methylation in Newborn Cord Blood

23% overall as compared to all CpGs on the array, p value = 2.8×10^{-100}), enhancers (29% versus 22% overall, p value = 5.7×10^{-45}), and DNase hypersensitivity sites (14% versus 12% overall, p value = 2.8×10^{-7}). Conversely, we found relative depletion in CpG islands (18% versus 31% overall, p value = 9.1×10^{-116}), FANTOM promoters (2.5% versus 6.7% overall, p value = 2.1×10^{-49}), and promoter-associated regions (13% versus 19% overall, p value = 2.3×10^{-33}). There was no statistically significant enrichment or depletion of sites mapping to imprinted DMRs (0.082% versus 0.16% overall, p value = 0.107).

Pathway Analysis

Our pathway analyses indicated that the FDR-significant CpG sites corresponded to genes enriched for several categories of biological processes, including anatomical development, phosphate-containing compound metabolism, nervous system development, and cell communication

processes (Figure S2). Based on DAVID, eight biological processes were enriched, including GTPase signal transduction, neuronal differentiation, and protein kinase activity (Figure S3). The top statistically significantly enriched diseases and biofunctions identified through Ingenuity software included tumor adhesion, neuron development, and connective tissue differentiation (Figure S4).

Functional Network Analysis

Functional network analysis revealed 447 significantly enriched GO terms after FDR correction was applied (q value < 0.01 for this analysis, Table S9). The majority of the enriched terms, and particularly the most statistically significant ones, pointed toward biological processes related to cell, tissue, or organ development, proliferation, morphogenesis, differentiation, growth, and other biologically relevant processes. There were also several enriched processes related to embryonic morphogenesis or development.

Table 2. Meta-analysis Results from Newborns for Selected Loci Not Previously Reported with Genome-wide Statistically Significant Differential Methylation in Newborn DNA in Relation to Sustained Maternal Smoking in Pregnancy

Chr.	Position	CpG	Mapped Gene ^a	Nearest Gene (10 Mb) ^b	Gene Group ^c	Regression Coefficient	SE	p Value	Direction of Effect across Cohorts ^d	Mean Beta ^e
1	24648203	cg06376426	<i>GRHL3</i>	<i>GRHL3</i>	TSS1500; body	-0.004	0.001	1.84E-04	-+-----	0.262
2	43685377	cg20629315	<i>THADA</i>	<i>THADA</i>	body	0.003	0.001	4.62E-04	-----++	0.896
2	206628553	cg22308949	<i>NRP2</i>	<i>NRP2</i>	body	-0.016	0.002	7.83E-12	-----	0.413
2	206628625	cg05348875	<i>NRP2</i>	<i>NRP2</i>	body	-0.026	0.004	1.13E-10	-----	0.613
2	206628692	cg14157435	<i>NRP2</i>	<i>NRP2</i>	body	-0.028	0.004	1.61E-10	-----	0.413
2	206692685	cg14400541	-	<i>NRP2</i>	-	-0.008	0.002	5.19E-05	+-----	0.501
3	189348936	cg05129081	<i>TP63</i>	<i>TP63</i>	TSS1500	0.012	0.002	1.21E-07	+++++--++	0.539
3	189349021	cg06720722	<i>TP63</i>	<i>TP63</i>	TSS200	0.009	0.002	8.49E-06	+++++--++	0.798
4	10117479	cg22821355	<i>WDR1</i>	<i>WDR1</i>	body	-0.007	0.002	1.50E-04	-----	0.382
4	81109888	cg01789499	<i>PRDM8</i>	<i>PRDM8</i>	5' UTR	-0.009	0.002	1.86E-04	-----+--	0.852
4	81110205	cg09595050	<i>PRDM8</i>	<i>PRDM8</i>	5' UTR	-0.018	0.004	1.71E-06	-----	0.739
4	81110459	cg14197071	<i>PRDM8</i>	<i>PRDM8</i>	5' UTR	-0.021	0.005	5.33E-06	-----+--	0.723
4	81111177	cg27111250	<i>PRDM8</i>	<i>PRDM8</i>	5' UTR	-0.020	0.005	1.61E-05	-----+--	0.708
4	81111393	cg27639662	<i>PRDM8</i>	<i>PRDM8</i>	5' UTR	-0.016	0.004	3.33E-05	-----+--	0.702
4	81117647	cg05452645	<i>PRDM8</i>	<i>PRDM8</i>	TSS1500; 5' UTR	-0.022	0.004	8.95E-09	-----+--	0.520
4	81117665	cg00138041	<i>PRDM8</i>	<i>PRDM8</i>	TSS1500; 5' UTR	-0.021	0.004	1.39E-06	-----+--	0.556
4	81117853	cg06373870	<i>PRDM8</i>	<i>PRDM8</i>	TSS1500; 5' UTR	-0.017	0.003	1.24E-08	-----+--	0.422
4	81118188	cg03463411	<i>PRDM8</i>	<i>PRDM8</i>	TSS1500; 5' UTR	-0.014	0.003	1.09E-06	-----+--	0.372
4	81118343	cg04235768	<i>PRDM8</i>	<i>PRDM8</i>	TSS1500; 5' UTR	-0.014	0.002	1.35E-09	-----+--	0.159
4	81118588	cg26299084	<i>PRDM8</i>	<i>PRDM8</i>	5' UTR; TSS200	-0.012	0.003	1.98E-06	-----+--	0.247
4	81118794	cg06307913	<i>PRDM8</i>	<i>PRDM8</i>	5' UTR; 1 st exon	-0.020	0.003	3.72E-09	-----+--	0.424
4	81119178	cg27242132	<i>PRDM8</i>	<i>PRDM8</i>	5' UTR	-0.022	0.004	2.98E-09	-----+--	0.240
4	81119198	cg18073471	<i>PRDM8</i>	<i>PRDM8</i>	5' UTR	-0.018	0.003	1.21E-08	-----+--	0.178
4	81119249	cg02458885	<i>PRDM8</i>	<i>PRDM8</i>	5' UTR	-0.010	0.002	5.94E-06	-----+--	0.189
4	81119299	cg11388320	<i>PRDM8</i>	<i>PRDM8</i>	5' UTR	-0.023	0.004	1.12E-08	-----	0.324
4	81119473	cg22902505	<i>PRDM8</i>	<i>PRDM8</i>	5' UTR	-0.027	0.004	1.21E-10	-----+--	0.433
4	81122726	cg05522011	<i>PRDM8</i>	<i>PRDM8</i>	body	-0.015	0.004	2.00E-04	-----+--	0.799
5	78365647	cg01856645	<i>DMGDH</i> ; <i>BHMT2</i>	<i>BHMT2</i>	TSS200; body	0.008	0.002	3.35E-06	+++++--++	0.177
5	78365687	cg06501366	<i>BHMT2</i> ; <i>DMGDH</i>	<i>BHMT2</i>	body; TSS1500	0.018	0.003	1.11E-10	+++++--++	0.408
5	78365691	cg08328513	<i>BHMT2</i> ; <i>DMGDH</i>	<i>BHMT2</i>	body; TSS1500	0.017	0.003	3.94E-09	+++++--++	0.265
5	78365710	cg23911707	<i>BHMT2</i> ; <i>DMGDH</i>	<i>BHMT2</i>	body; TSS1500	0.006	0.001	5.69E-06	+++++--++	0.260
5	78365801	cg03400060	<i>BHMT2</i> ; <i>DMGDH</i>	<i>BHMT2</i>	body; TSS1500	0.012	0.002	2.96E-10	+++++--++	0.392
5	78366076	cg01902605	<i>BHMT2</i> ; <i>DMGDH</i>	<i>BHMT2</i>	body; TSS1500	0.013	0.002	1.50E-09	+++++--++	0.707
6	7673306	cg25370658	-	<i>BMP6</i>	-	0.004	0.001	2.63E-04	+++--++	0.806
6	7698374	cg17951878	-	<i>BMP6</i>	-	0.013	0.003	1.15E-06	+++++--++	0.286
6	7731280	cg23623251	<i>BMP6</i>	<i>BMP6</i>	body	0.006	0.002	3.25E-04	+++++--++	0.783
6	10405499	cg16199280	<i>TFAP2A</i>	<i>TFAP2A</i>	body	0.006	0.002	3.26E-04	+-----	0.342

(Continued on next page)

Table 2. Continued

Chr.	Position	CpG	Mapped Gene ^a	Nearest Gene (10 Mb) ^b	Gene Group ^c	Regression Coefficient	SE	p Value	Direction of Effect across Cohorts ^d	Mean Beta ^e
6	55767865	cg16728651	–	<i>BMP5</i>	–	–0.010	0.002	1.05E-06	–	0.729
6	152011415	cg08161546	<i>ESR1</i>	<i>ESR1</i>	TSS1500	0.008	0.002	3.50E-04	+++++	0.709
6	152124815	cg08415493	<i>ESR1</i>	<i>ESR1</i>	5' UTR	–0.003	0.001	1.74E-04	–	0.706
6	152126736	cg20893956	<i>ESR1</i>	<i>ESR1</i>	5' UTR; TSS200	–0.009	0.002	4.13E-05	–+–	0.620
6	152126785	cg07746998	<i>ESR1</i>	<i>ESR1</i>	5' UTR; TSS200	–0.006	0.002	1.18E-04	–+–	0.594
6	152126895	cg21157690	<i>ESR1</i>	<i>ESR1</i>	5' UTR; 1 st exon	–0.008	0.002	5.70E-05	–+–	0.747
6	152126938	cg17264271	<i>ESR1</i>	<i>ESR1</i>	5' UTR; 1 st exon	–0.009	0.002	1.26E-06	–+–	0.627
6	152130058	cg04063345	<i>ESR1</i>	<i>ESR1</i>	body	–0.013	0.004	1.22E-04	–+–	0.507
6	152130207	cg15626350	<i>ESR1</i>	<i>ESR1</i>	body	–0.018	0.004	1.42E-06	–+–	0.444
6	152130332	cg00601836	<i>ESR1</i>	<i>ESR1</i>	body	–0.014	0.003	1.19E-06	–+–	0.676
8	1403050	cg16442298	–	<i>DLGAP2</i>	–	–0.004	0.001	3.28E-04	–	0.716
8	1404023	cg03551508	–	<i>DLGAP2</i>	–	–0.007	0.002	2.74E-05	–	0.746
8	1427491	cg00827210	–	<i>DLGAP2</i>	–	–0.007	0.001	4.83E-07	–+–	0.882
8	1442292	cg13063207	–	<i>DLGAP2</i>	–	–0.006	0.001	1.85E-05	–	0.847
8	1458508	cg24526596	<i>DLGAP2</i>	<i>DLGAP2</i>	5' UTR	–0.005	0.001	2.52E-04	–+–	0.590
8	1462903	cg25955692	<i>DLGAP2</i>	<i>DLGAP2</i>	5' UTR	–0.005	0.001	1.67E-05	++–	0.874
8	1468625	cg00598912	<i>DLGAP2</i>	<i>DLGAP2</i>	5' UTR	–0.003	0.001	1.39E-04	–	0.831
8	1494546	cg23424125	<i>DLGAP2</i>	<i>DLGAP2</i>	5' UTR	–0.010	0.003	3.23E-04	–+–	0.850
8	1501226	cg03185622	<i>DLGAP2</i>	<i>DLGAP2</i>	body	–0.005	0.001	5.33E-07	+–+–	0.825
8	1526540	cg15833940	<i>DLGAP2</i>	<i>DLGAP2</i>	body	–0.013	0.003	6.70E-06	–	0.659
8	1534376	cg02840179	<i>DLGAP2</i>	<i>DLGAP2</i>	body	–0.004	0.001	1.05E-04	–	0.816
8	1615080	cg02709139	<i>DLGAP2</i>	<i>DLGAP2</i>	body	–0.007	0.002	1.32E-05	–+–	0.870
8	1616381	cg04687241	<i>DLGAP2</i>	<i>DLGAP2</i>	body	–0.008	0.002	6.92E-06	–+–	0.666
8	1618448	cg06040034	<i>DLGAP2</i>	<i>DLGAP2</i>	body	–0.013	0.003	2.42E-06	–	0.619
8	1649758	cg02083412	<i>DLGAP2</i>	<i>DLGAP2</i>	3' UTR	–0.004	0.001	3.15E-05	–+–	0.129
8	1649868	cg22763586	<i>DLGAP2</i>	<i>DLGAP2</i>	3' UTR	–0.013	0.003	5.83E-07	–+–	0.450
8	1650172	cg27351978	<i>DLGAP2</i>	<i>DLGAP2</i>	3' UTR	–0.015	0.004	8.69E-05	–+–	0.566
8	1650309	cg02690013	<i>DLGAP2</i>	<i>DLGAP2</i>	3' UTR	–0.013	0.003	9.92E-06	–+–	0.599
14	54412780	cg23104439	–	<i>BMP4</i>	–	0.005	0.001	2.08E-04	+++++	0.741
14	54418728	cg05928290	<i>BMP4</i>	<i>BMP4</i>	body	0.024	0.003	1.48E-19	+++++	0.759
14	54418804	cg05923197	<i>BMP4</i>	<i>BMP4</i>	body	0.029	0.003	1.08E-18	+++++	0.699
14	54418851	cg09367901	<i>BMP4</i>	<i>BMP4</i>	body	0.019	0.002	3.98E-17	+++++	0.827
14	54419614	cg08046044	<i>BMP4</i>	<i>BMP4</i>	5' UTR	0.005	0.001	2.70E-09	+++–	0.077
14	54424149	cg24526899	<i>BMP4</i>	<i>BMP4</i>	TSS1500	0.007	0.002	5.14E-04	+++++	0.441
17	76930245	cg04999637	–	<i>TIMP2</i>	–	0.005	0.001	6.91E-05	+++++	0.583

Meta-analysis results of the association between sustained maternal smoking during pregnancy and DNA methylation in newborns, adjusted for covariates, using normalized methylation betas as the outcome. Selected not previously reported loci genome-wide significant after FDR correction. Results sorted by the chromosome (chr.) and position of the CpG sites listed. Selection limited to genes prioritized for discussion.

^aUCSC Genome Browser annotated gene.

^bNearest gene (within 10 Mb) symbol, determined with the Snipper program.

^cUCSC gene region feature category. Regions for the gene and related isoforms are listed.

^dDirection of effect across cohorts included in the statistical model: maternal smoking during pregnancy associated with increased (+) or decreased (–) methylation in alphabetical order of cohorts.

^eAverage of the mean methylation beta values across the newborn cohorts. For complete listing of CpGs differentially methylated in relation to sustained maternal smoking during pregnancy and for results from meta-analysis models unadjusted for covariates and adjusted for covariates and cell type, see Table S3.

Methylation Transcription Analysis

To assess transcriptional effects related to methylation differences, we investigated whether methylation status correlated with gene expression levels for our 2,965 CpGs associated with sustained maternal smoking in newborns that we identified through literature review as not having been previously reported. In the Rotterdam Study dataset of adults, out of the 2,636 (of the 2,965) CpGs that we were able to match to a gene transcript (± 250 kb), 254 unique CpGs (343 total CpG-gene transcript associations) were significantly associated with expression of a nearby gene in whole blood from adults (FDR p value < 0.05 , [Table S10](#)). We observed strong associations for several CpGs annotated to the same gene and corresponding gene expression levels, most strikingly for *IL32* (MIM: 606001), which had four CpGs associated with its expression, and *HOXB2* (MIM: 142967), which had several CpGs related to its expression (lowest p value 2.38×10^{-72} , [Table S10](#)). In the much smaller study of children at age four from INMA ($n = 107$), 35 CpGs were associated with gene expression (FDR p value < 0.05). The following six genes had CpGs with methylation that was statistically significantly related to gene expression in both the Rotterdam Study adults and INMA children: *ENOSF1* (MIM: 607427), *HOXB2* (MIM: 142967), *IL32* (MIM: 606001), *NLRP2* (MIM: 609364), *PASK* (MIM: 607505), and *TDRD9*. In both the adult and child datasets, for the majority of CpGs statistically significantly associated with expression, the direction was inverse (higher methylation, lower expression). This inverse relationship represented 68% (Clopper-Pearson 95% confidence interval = 62%–73%) of the adult associations and 79% (Clopper-Pearson 95% confidence interval = 62%–91%) of the associations for children.

Newborn DNA Methylation Related to Any Maternal Smoking during Pregnancy

In addition to the sustained smoking model, we meta-analyzed the effect of any maternal smoking during pregnancy on newborn methylation. As expected, based on previous literature,¹² we found that despite the much larger number of women with any smoking during pregnancy, there were fewer statistically significant findings for this less specific exposure (4,653 FDR-significant CpGs, [Table S3](#)).

Discussion

We combined data across studies in a large-scale epigenome-wide meta-analysis to evaluate the association between maternal smoking during pregnancy and DNA methylation in offspring. We established the PACE consortium to study this association and used 13 birth cohort studies from the US and Europe that, with the same reproducible platform, measured CpG-specific DNA methylation across the epigenome in newborns. Combining these

studies resulted in the discovery of 6,073 statistically significant CpGs; 3,932 remained statistically significant after adjustment for cell type proportion. Our results are remarkably robust to different modeling techniques. Our findings were very similar when using either the raw methylation betas or the normalized betas as the outcome. This is despite the variety of data processing methods used across the cohorts for normalization and corrections for technical variables such as batch (described in the [Supplemental Note](#)). This consistency is reassuring given the range of published methods available for researchers to apply to 450K DNA methylation data for quality control, normalization, and adjustment for technical variation. Furthermore, our main findings persisted after cell type adjustment ([Table S3](#), [Figure S1](#)).

As predicted based on earlier evaluation of top findings for maternal smoking in the MoBa cohort,¹² we had fewer statistically significant findings for any smoking during pregnancy than for sustained smoking during pregnancy ([Figure S5](#)). Nonetheless, with the large sample size of this meta-analysis, we still observed many statistically significant CpGs after FDR correction in the any smoking models, and the directions of effect and p values were similar to those from the sustained smoking models ([Table S3](#), [Figure S6](#)). However, the stronger signal for sustained smoking suggests that this might be the more powerful variable for studying epigenetic effects and possible health outcomes from this exposure in offspring.

Our observation of a large number of genome-wide significant CpGs related to maternal smoking is not surprising given reports of multiple genome-wide significant loci identified in single studies, all with smaller sample sizes.^{7,8,10,22,37,38} Reassuringly, among our myriad findings, the top hit in all newborn models was *AHRR* cg05575921 (p value $< 1.64 \times 10^{-193}$), which has been observed as differentially methylated in relation to smoking in many studies of adults and children.^{7,8,10,28,35,53}

Our enrichment testing of the genome-wide results is in line with previous findings showing that island shores, enhancers, and DNase I hypersensitive sites are more dynamic (susceptible to methylation changes) than promoter regions⁵⁴ and imprinted loci.⁵⁵ These regions might be more resistant to changes in DNA methylation in response to in utero exposure.⁵⁵ Thus, it is not surprising that associations between maternal smoking and newborn methylation might be more likely to be found in island shore and enhancer regions as opposed to promoters or CpG islands.

To assess the underlying biology involved in the associated genomic regions, we applied pathway and functional analyses, as well as tests of enrichment. These results implicated numerous neurological pathways, pathways involved in embryogenesis, and various developmental pathways. These observations could provide insight into the etiology of childhood health outcomes related to maternal smoking during pregnancy.

We focus discussion on some specific genes among the associations that, according to our literature review, had

not been previously reported (2,965 CpGs annotating to 2,017 mapped or nearest genes). For 27 of these genes, mutations or SNPs have been implicated in susceptibility to orofacial clefts (as identified with the Snipper program described in the [Supplemental Note](#)). This includes the following genes (each representing one FDR-significant CpG unless otherwise specified): *BHMT2* ([MIM: 605932] six CpGs), *GRHL3* (MIM: 608317), *THADA* (MIM: 611800), *GAD67* (MIM: 605363), *TP63* ([MIM: 603273] two CpGs), *MSX1* (MIM: 142983), *WDR1* (MIM: 604734), *SPP1* (MIM: 166490), *BMP6* (MIM: 112266), *TFAP2A* (MIM: 107580), *COL11A2* ([MIM: 120290] three CpGs), *PDGFRA* (MIM: 173490), *MN1* (MIM: 156100), *MSX2* ([MIM: 123101] four CpGs), *PVT1* (MIM: 165140), *ZIC2* (MIM: 603073), *HOXA2* ([MIM: 604685] ten CpGs), *WNT3* (MIM: 165330), *RUNX2* ([MIM: 600211] two CpGs), *TERT* (MIM: 187270), *SPATA13* ([MIM: 613324] two CpGs), *VAX1* (MIM: 604294), *TIMP2* (MIM: 188825), *NOG* (MIM: 602991), *BEST3* (MIM: 607337), *MYH9* (MIM: 160775), and *BMP4* ([MIM: 112262] six CpGs) (results in [Table S5](#)). Although this does not imply that the smoking-related CpGs are on the causal pathway, we note that the Surgeon General's Report summarizes the evidence as sufficient to infer a causal relationship between maternal smoking during pregnancy and these birth defects.² Many of these genes also have varied biological effects relevant to other aspects of development.

Among this group of genes previously related to orofacial clefts, bone morphogenetic protein 4 (*BMP4*) is especially interesting. Maternal smoking might interact with SNPs in this gene in relation to oral clefts.⁵⁶ We identified six CpGs in *BMP4* at genome-wide significance in newborns; two remained statistically significant in the older children. In addition to orofacial clefts, SNPs in *BMP4* are related to tooth development and eruption, as well as to colorectal cancer in genome-wide association studies (GWASs).⁵⁷ BMPs, including *BMP4*,⁵⁸ also play an important role in lung development: reduced lung function among infants is an established consequence of maternal smoking during pregnancy.² A plot showing greater detail on the CpGs in or near *BMP4* is provided in [Figure S7](#).

We observed six CpGs significantly related to maternal smoking in betaine-homocysteine methyltransferase (*BHMT2*; [Figure S8](#)). Genetic variants in this gene have been associated with orofacial clefts in candidate-gene studies⁵⁹ and with selenium levels in GWASs.^{60,61} Of note, in experimental studies, selenium has been shown to protect against orofacial clefts induced by exposure to teratogens.⁶² In the Cancer Genome Atlas, methylation of *BHMT2* in lung adenocarcinoma (lung cancer [MIM: 211980]) was strongly correlated (3rd rank genome wide) with smoking history.⁶³

The gene *PRDM8* (PR domain containing 8 [MIM: 616639]) has the largest number of CpGs (18 of 61, based on Illumina annotation) significantly associated with maternal smoking during pregnancy. Maternal smoking during pregnancy was associated with decreased methyl-

ation throughout the gene. *PRDM8* is one of several *PRDMs* belonging to the SET domain family of histone methyltransferases.⁶⁴ *PRDM* genes either act as direct histone methyltransferases or recruit a suite of histone-modifying enzymes to target promoters.⁶⁵ *PRDM8* specifically methylates H3K9 of histones to repress transcriptional activity.⁶⁶ *PRDM8* expression is tightly regulated in a spatiotemporal manner during neural development;⁶⁷ it regulates morphological transition in neocortical development⁶⁸ and forms part of a repressor complex that directs, through regulation of cadherin-11, neural circuit assembly.⁶⁹ Thus *PRDM8* appears to play an important role in neurologic development.

DLGAP2 (discs large homolog-associated protein 2 [MIM: 605438]) is another gene with a large number of significant CpGs (14 of 192 tested) associated with maternal smoking in our study. *DLGAP2*, also known as *SAPAP2*, belongs to a gene family that encodes SAP90/PSD95-associated proteins (SAPAPs), and is known to be involved in the molecular organization of synapses and in neuronal cell signaling.⁷⁰ *DLGAP2* was first identified in studies of progressive epilepsy with mental retardation (EPMR [MIM: 610003])⁷¹ and has been associated with other CNS disorders such as schizophrenia (SCZD [MIM: 181500])⁷² and autism spectrum disorders (ASD [MIM: 209850]).^{73,74} Differential methylation at this locus in a rat model appears to play a role in the development of post-traumatic stress disorder.⁷⁵

The neuropilin-2 (*NRP2* [MIM: 602070]) gene had three CpGs located in close proximity (among 48 tested) that were statistically significantly associated with maternal smoking during pregnancy. *NRP2* is one of two transmembrane receptors for axonal guidance cues of the class 3 semaphorin (SEMA) family and is expressed in sympathetic neural crest cells and their progeny.⁷⁶ It might also be required in vivo for sorting migrating cortical and striatal interneurons to their correct destination.⁷⁷ *NRP2* also functions as a receptor for some forms of vascular endothelial growth factor, thereby playing a crucial role in angiogenesis and lymphangiogenesis.⁷⁸ Polymorphisms in *NRP2* have been associated with several diseases, including autism⁷⁷ and multiple cancers.^{79–83}

Hypermethylation of *ESR1* (estrogen receptor 1 [MIM: 133430], a key nuclear transcription factor) on chromosome 6q25.1 is well-studied in relation to presence and prognosis of various malignancies such as breast cancer and hepatocellular carcinoma,^{84,85} as well as asthma.^{86,87} We found an inverse association between maternal smoking and methylation levels for seven out of the eight FDR-significant *ESR1* CpGs. *ESR1* hypomethylation has been reported in relation to induced microRNA expression (synthetic miR-29b oligonucleotides) in acute myeloid leukemia cells.⁸⁸

To evaluate possible functional gene expression effects of methylation at the CpGs that we found to be significantly related to maternal smoking, we analyzed data from two studies—one of adults and another of children

at age four years. Although on first pass, one might expect a higher proportion of the CpGs related to maternal smoking to also be related to gene expression, there are several factors that decrease the likelihood of seeing significant associations. Most importantly, the sample size for discovery of the methylation association with smoking was much larger than that of the datasets available to correlate gene expression and methylation (about 10-fold smaller for the adult gene expression dataset and about 60-fold smaller for the childhood dataset). In addition, gene expression in blood might be more transient than methylation, decreasing the ability to find significant associations with a single gene-expression measurement. Furthermore, constitutive gene expression is measured in this setting, whereas many genes are inducible and methylation might contribute to this process. Lastly, some in-utero-induced changes to methylation could have affected transcription during fetal development but not in postnatal life, and might have transcription-independent functional mechanisms. Nonetheless, we observed significant associations between methylation and gene expression at six genes in both the adults and the children. The majority of CpGs significantly associated with expression were in the commonly expected direction of methylation related to gene silencing. Notably, CpGs in *IL32* (Figure S9), a proinflammatory cytokine involved in several diseases such as asthma⁸⁹ and cancer,⁹⁰ *HOXB2*, a transcription factor involved in development⁹¹ and several cancer forms,⁹² and *PASK* (PAS domain containing serine/threonine kinase), involved in glucose homeostasis,⁹³ were significantly associated with expression in both datasets.

We analyzed the associations of CpGs with expression levels of genes within a region of 250 kb up- or downstream of the CpG. Consensus on the optimal physical distance for these analyses is lacking. However, in a recent study, associations between CpGs and SNPs were the strongest when within close proximity (500 kb) of the CpG.⁴⁸ Despite the limitations with the expression datasets included in our study, we believe that the transcriptomics data provide functional support for our maternal smoking findings.

In older children, all of the CpGs significantly associated with maternal smoking in newborns gave at least nominal levels of significance (p value < 0.05). This skew of the distribution of p values toward small values was much more than expected by chance (Kolmogorov p value $< 2.2 \times 10^{-16}$), demonstrating a very high level of replication and persistence of findings at birth into later childhood. This is consistent with and substantially extends a few previous reports.^{9,12} We had only very limited data with repeat measures in the same individuals so we could not meta-analyze change in methylation over time.

This inaugural paper from the PACE consortium represents a major effort to combine data from many studies in a large-scale meta-analysis of epigenome-wide association studies of maternal smoking in relation to methylation in newborns. We report at least an order of

magnitude more genes differentially methylated in response to maternal smoking than have been identified in any previous study. This suggests that meta-analysis in epigenome-wide association studies produces similar success to that of genome-wide association SNP studies in the identification of biologically meaningful loci. The similarity in the results obtained when using the raw betas compared to those obtained when using normalized betas generated with various methods indicates that cohort-specific processing methods do not interfere with the ability to perform meta-analysis.

We identified nearly 3,000 CpGs corresponding to genes differentially methylated in offspring in relation to whether their mothers smoked during pregnancy. Some of these genes have been implicated in genetic studies of orofacial clefts or asthma, both conditions related to maternal smoking in pregnancy, and others in the pathogenesis of cancers that are associated with adult smoking, including lung, colorectal (CRC [MIM: 114500]), and liver (HCC [MIM: 114550]).² We also find substantial persistence of effects of maternal smoking identified in newborns into later childhood. Our findings might implicate epigenetic mechanisms in the etiology of these exposure-disease relationships. This large-scale study also provides confirmation of previously reported loci, many of which have not been previously replicated. Pathway analysis highlights the involvement of identified genes in various developmental pathways, and functional effects at the transcriptomics level were observed for many of the identified CpGs. These findings could provide new insights into the mechanisms involved in the detrimental health outcomes that arise from this important in utero exposure.

Supplemental Data

Supplemental Data include a Supplemental Note, nine figures, and ten tables and can be found with this article online at <http://dx.doi.org/10.1016/j.ajhg.2016.02.019>.

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Web Resources

The URLs for data presented herein are as follows:

A Catalog of Published Genome-Wide Association Studies, National Human Genome Research Institute, <http://www.genome.gov/gwastudies>

Bioconductor, <http://www.bioconductor.org/>

Infinium HumanMethylation450K v1.2 Product Files, http://support.illumina.com/downloads/infinium_humanmethylation450_product_files.html

Ingenuity, www.ingenuity.com
 OMIM, <http://www.omim.org/>
 Snipper, <http://csg.sph.umich.edu/boehnke/snipper>
 The R Project for Statistical Computing, R v.3.0.2, <http://www.r-project.org/>
 UCSC Human Genome Browser, <http://hgdownload.soe.ucsc.edu/goldenPath/hg19/database/>

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